

PERIVASCULAR MESENCHYMAL PRECURSOR CELL INDUCED BLOOD VESSEL FORMATION

5 FIELD OF THE INVENTION

This invention relates to mesenchymal precursor cells that carry a perivascular marker and are able to induce blood vessel formation, to a composition comprising such precursor cells and a method of inducing blood vessels. The invention also relates to treatment of cardiovascular conditions in particular ischemia.

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BACKGROUND OF THE INVENTION

Vascular conditions constitute a major health problem, particularly in western countries.

Vascular conditions include heart conditions, peripheral vascular disease and cerebrospinal vascular conditions.

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A major proportion of these conditions result from a lack of supply of blood to the respective tissues which thus are either chronically or acutely deprived of necessary levels of oxygen and nutrients. Typically these conditions result in ischemias where there has been blockage of the blood vessel by build up of, for example, plaque or physical blood vessel damage such that 20 these are either blocked or constricted.

Heart conditions are perceived as the most prominent vascular disease. About 11 million adults in the United States of America (1995) had coronary heart disease, that however is out of a total of about 60 million adults with cardiovascular disease. Other vascular conditions 25 thus affect a greater number of adults.

A relatively common cerebrovascular condition can manifest as a stroke, where an occlusion can lead to an acute attack. A gradual diminution of supply may also lead to decreased capacity of the brain to function and it has been postulated that such conditions may be 30 associated with the onset of certain dementias.

Peripheral vascular disease are associated with a number of conditions, for example, as a complication of diabetes where a typical inadequacy of the microcirculation depeletes supply to the extremities particularly the feet and legs of oxygen and nutrients.

5 Another example of reduced localized supply can occur with the treatment of various wounds including severe burns or chronic wounds such as bed sores. Chronic wounds are difficult to heal, partly due to an insufficient vascular bed supply of nutrient and healing compounds.

Scar formation may also be exacerbated because the healing process often involves a highly

10 fibrotic tissue forming within minimal blood vessel formation. Scar formation is also a difficulty associated with achieving an adequate vascular supply in circumstances where a prosthesis or other implant is surgically position in a human tissue. An inadequate blood supply to the interface between the implant and the surrounding tissue can lead to medical complications and necrosis. This is of far more noticeable relevance where the implant is
15 intended as a long term slow release depot of for example a pharmaceutical.

Treatment of myocardial ischemias are probably the most advanced of current treatments of vascular conditions. Present treatments include pharmacological therapies, coronary artery bypass surgery and percutaneous revascularization using techniques such as balloon

20 angioplasty. Standard pharmacological therapy to aims either increase blood supply to the heart muscle or decreasing the demand of the heart muscle for oxygen and nutrients. Increased blood supply to the myocardium by relaxation of smooth muscle is achieved by administering agents such as calcium channel blockers or nitroglycerin. Decreased demand of the heart muscle for oxygen and nutrients is accomplished either by agents that decrease the

25 hemodynamic load on the heart, such as arterial vasodilators, or those that decrease the contractile response of the heart to a given hemodynamic load, such as β -adrenergic receptor antagonists. Surgical treatment of ischemic heart disease is based on the bypass of diseased arterial segments with strategically placed bypass grafts. Percutaneous neovascularization is based on the use of catheters to reduce the narrowing in diseased coronary arteries. All of
30 these strategies are used to decrease the number of, or to eradicate, ischemic episodes, but all have various limitations, and particularly the pharmaceutical approach can have severe side effects.

Preliminary reports describe new vessel development in the heart through the direct injection of angiogenic proteins or peptides. The several members of the fibroblast growth factor (FGF) family (namely acidic fibroblast growth factor, aFGF; basic fibroblast growth factor, bFGF; 5 fibroblast growth factor-5, FGF-5 and others) have been implicated in the regulation of angiogenesis during growth and development. Gene therapy has been suggested by Hammond et al in US 5792453 as a delivery mechanism for these angiogenic compounds.

Another suggested approach to promoting new blood vessel formation for treatment of 10 vascular conditions is the administration of stem cells which can differentiate and give rise to cells required for such blood vessels to form. One problem associated with this approach is that it is not entirely clear which progenitor cells are responsible for formation of blood vessel, or whether indeed more than one cell type is required or whether other angiogenesis promoters are required.

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One reported approach described in US patent no 5980887 (to Isner et al) has resulted from the isolation of an endothelial progenitor cell and the discovery that such cells play a role in blood vessel formation.

20 Numerous attempts at isolating and enriching mesenchymal precursor cells have been made because of the potential that these cells have for medicinal use. Pitterer *et al.*, (1999) show the expansion of clonogenic cells from bone marrow and describes a preparation of enlarged mesenchymal stem cells. A more recent example of such a method providing for a relatively high yield from bone marrow is disclosed in publication WO01/04268 to Simmons *et al.*, 25 Neither of these reported mesenchymal cells were indicated as being capable of regeneration vascular lineages of cells capable of leading to blood vessel formation.

To date however there have been no examples of isolated mesenchymal precursor cells capable of forming vascular tissues *in vivo*.

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SUMMARY OF THE INVENTION

The present invention arises from the finding that a population of mesenchmal precursor cells (MPCs) is present in a perivascular niche. This has led to the demonstration that there is a much wider range of tissue type sources of MPCs than the single tissue, bone marrow, referred to in WO01/04268. The present invention arises from the additional finding that an enriched 5 population MPCs can be differentiated into two populations discriminated by the marker 3G5. MPCs that are 3G5 positive are considered of interest particularly for neovascularization applications, although demonstrably they are also able to differentiate into other tissue types. It is an additional finding of the present invention that levels of MPCs present in preferred enriched populations of this invention are able to give rise to sufficient numbers of committed 10 cells to provide a number of differentiated tissue types. It is an additional finding of the present invention that certain levels of MPCs are useful on introduction into a patient to provide a measurable vascularisation benefit. It has thus specifically beenfound that a level of an estimated about 10^5 MPCs are sufficient to provide a measurable benefit of cardiac improvement in an ischemic rat myocardium. This then provides a datum from which an 15 assessment can bemade about the numbers of MPCs required to provide a beneficial effect. This is also believed to be the first time that a cardiac benefit has been shown on administration of a mesenchymal precursor cell to the heart.

In a first form of a first aspect the invention might be said to reside in a method of inducing 20 the formation or repair of blood vessels in a target tissue of a patient, the method comprising the steps of administering to said patient an effective amount of a population of enriched perivascular mesenchymal precursor cells (MPCs) to induce new blood vessel formation in target tissue.

25 In a first form of a second aspect the invention might be said to reside in a method of repairing damaged tissue in a human subject in need of such repair, the method comprising:
(a) obtaining an enriched population of MPC, and
(b) contacting an effective amount of the enriched population of MPC with the damaged tissue of said subject

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In a first form of a third aspect the invention might be said to reside in a method of repairing damaged tissue in a human subject in need of such repair, the method comprising:

(a) expanding the enriched MPC of claim 41 in culture, and
(b) contacting an effective amount of the expanded cells with the damaged tissue of said subject.

5 In a first form of a fourth aspect the invention might be said to reside in a method of inducing formation or repair of blood vessels, the method comprising the steps of providing a population of enriched perivascular mesenchymal precursor cells (MPCs), contacting said cells with a growth media, and culturing said cells under conditions to induce them to differentiate into new blood vessels.

10 In a first form of a fifth aspect the invention might be said to reside in a composition for use in inducing heart vessel formation comprising a population of mesenchymal precursor cells (MCPs) in a pharmaceutically acceptable carrier, said MPCs carrying a perivascular marker and being a vascular progenitor.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Properties of STRO-1⁺ MACS-isolated cells co-labeled with anti-CD146 (CC9). (A) Sort region, R1, represents the double positive STRO-1^{BRT}/CD146⁺ population. (B) The incidence of clonogenic cell colonies (>50 cells) based on STRO-1^{BRT}/CD146⁺ expression was determined by limiting dilution analysis of 24 replicates per cell concentration using Poisson distribution analysis from 5 independent experiments. Forward (size) and perpendicular (granularity) light scatter characteristics of BMMNCs (C), STRO-1^{int}/CD146⁻ cells (D) and STRO-1^{BRT}/CD146⁺ cells (E). (F) RT-PCR analysis of STRO-1^{BRT}/CD146⁺ sorted marrow cells for CBFA1 (lane 2), osteocalcin (lane 4) and GAPDH (lane 6) transcripts. Control cells (BMSSC cultures grown in the presence of dexamethasone) expressing CBFA1 (lane 1), osteocalcin (lane 3), and GAPDH (lane 5) is also shown. Reaction mixes were subjected to electrophoresis on a 1.5% agarose gel and visualised by ethidium bromide staining. (G) *In situ* expression of CD146 on blood vessel (bv) walls (arrow) in human bone marrow

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(bm) sections near the bone (b) surface 20X. Sections were counter stained with Hematoxylin. (H) Dual Immunofluorescence staining demonstrating reactivity of the STRO-1 antibody labeled with Texas red and the CC9 antibody labeled with fluorescein isothiocyanate, reacting to blood vessel walls in frozen sections of human bone marrow.

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Figure 2. Immunophenotypic analysis of DPSCs *in vivo*. The bar graph depicts the number of clonogenic colonies retrieved from single cell suspensions of dental pulp following immunomagnetic bead selection based on reactivity to antibodies that recognize STRO-1, CD146, and 3G5 and isotype-matched negative control antibodies. The data are expressed as the number of colony-forming units obtained in the bead positive cell fractions as a percentage of the total number of colonies in unfractionated pulp cells averaged from three separate experiments. Statistical significance (*) was determined using the student t-test (p 0.01) comparing the percent total number of colonies for each antibody with the corresponding isotype-matched control.

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Figure 3. Reactivity of perivascular makers in dental pulp. (A) Immunolocalization of the STRO-1 antigen on blood vessels (small arrows) in human dental pulp (p) and around perineurium (large arrow) surrounding a nerve bundle (nb) 20X. (B) Dual Immunofluorescence staining demonstrating reactivity of the STRO-1 antibody labeled with Texas Red to dental pulp perineurium (arrow) in combination with an anti-neurofilament antibody labeled with fluorescein isothiosyanate staining the inner nerve bundle (nb), 40X. (C) Immunolocalization of the CD146 antigen to blood vessel walls in human dental pulp tissue 20X. (D) Dual Immunofluorescence staining demonstrating reactivity of the STRO-1 antibody labeled with Texas red to a blood vessel and the CC9 antibody labeled with fluorescein isothiosyanate. (E) Immunohistochemical staining of pulp tissue with a rabbit polyclonal anti-DSP antibody (arrow) to the odontoblast outer layer (od). 20X. (F) 3G5 reactivity to a single pericyte (arrow) in a blood vessel (bv) wall 40X. Tissue sections were counter stained with Hematoxylin.

Figure 4. 3G5 reactivity to BMSSCs. (A) The representative histogram depicts a typical dual-color FACS analysis profile of whole bone marrow mononuclear cells (BMMNCs) expressing CD146 (PE) and 3G5 (FITC). (B) Colony efficiency assays were performed for all the different expression patterns observed (regions "R" 1-6). The data are expressed as the mean incidence of colony-forming units for each cell fraction averaged from three separate experiments.

Figure 5. Developmental potential of purified BMSSCs and DPSCs *in vivo*. Cytospin preparations of MACS/FACS isolated STRO-1^{BRT}/CD146⁺ marrow cells (arrow) stained with an antibody specific to α -smooth muscle actin (A) and von Willebrand Factor (B). CD146⁺ pulp cells (large arrow) isolated by immunomagnetic bead selection (magnetic beads depicted by small arrows), stained with an antibody specific to α -smooth muscle actin (C) and von Willebrand Factor. (D). (E) Ectopic bone formation (b) and haematopoietic/adipogenic marrow (bm) by *ex vivo* expanded cells derived from STRO-1^{BRT}/CD146⁺ BMSSCs transplanted with HA/TCP into immunocompromised mice for three months (E). (F) Ectopic formation of dentin (d) and fibrous pulp tissue (p) by *ex vivo* expanded cells derived from CD146⁺ DPSCs transplanted with HA/TCP into immunocompromised mice for three months. Sections were stained with Hematoxylin & Eosin.

Figure 6 Expression of CD34, CD45 and Glycophorin-A on STRO-1 positive bone marrow mononuclear cells. Representative histograms depicting typical dual-colour flow cytometric analysis profiles of STRO-1 positive bone marrow mononuclear cells isolated initially by magnetic activated sorting and co-stained with antibodies directed against CD34 (A), CD45 (B) or Glycophorin-A (C). The STRO-1 antibody was identified using a goat anti-murine IgM-fluorescein isothiocyanate while CD34, CD45 and Glycophorin-A were identified using a goat anti-murine IgG- phycoerythrin. The high expressing STRO-1 fraction which contained the clonogenic MPC population was isolated by fluorescence activated cell sorting based on regions R1 and R2.

Figure 7 Bone marrow MPC are STRO-1 bright, CD34 negative, CD45 negative and Glycophorin-A negative. The graph depicts the results of in vitro adherent colony formation assays performed for each of the different sorted STRO-1 bright populations selected by their co-expression or lack of either the CD34, CD45 or Glycophorin-A antigens, based on regions R1 and R2 as indicated in Figure 6. These data are expressed as the mean incidence of colony-forming units for each cell fraction averaged from two separate experiments.

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10 Figure 8 Reactivity of perivascular makers in different human tissues. Dual-colour immunofluorescence staining demonstrating reactivity of (A) STRO-1 and CD146, (B) STRO-1 and alpha-smooth muscle actin, and (C) 3G5 and CD146, on blood vessels and connective tissue present on spleen, pancreas (Panel 1), brain, kidney (Panel 2), liver, heart (Panel 3) and skin (Panel 4) 20X. The 15 STRO-1 and 3G5 antibodies were identified using a goat anti-murine IgM-Texas Red while CD146 and alpha-smooth muscle actin were identified using a goat anti-murine IgG-fluorescein isothiocyanate. Co-localization is indicated by overlapping areas of yellow and orange fluorescence (white arrows).

20 Figure 9 Isolation of adipose-derived MPC by FACS. Representative flow cytometric histograms depicting the expression of STRO-1, CD146 and 3G5 in fresh 25 preparations of peripheral adipose-derived single-cell suspensions generated following collagenase/dispase digestion as previously described (Shi and Gronthos 2003). The antibodies were identified using either a goat anti-murine IgM or IgG-phycoerythrin. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth medium to assess the incidence of adherent colony-forming cells in each cell fraction.

30 Figure 10 Clonogenic adipose-derived MPC are positive for STRO-1/3G5/CD146. The bar graph depicts the number of clonogenic colonies retrieved from single cell suspensions of enzymatically digested human peripheral adipose tissue,

following fluorescence activated cell sorting, based on their reactivity to antibodies that recognize STRO-1, CD146, and 3G5 (Figure 9), then cultured in standard growth medium as previously described for bone marrow and dental pulp tissue (Shi and Gronthos 2003). The data are expressed as the number of colony-forming units obtained per 10^5 cells plated in the positive and negative cell fractions averaged from two separate experiments.

Figure 11 Immunophenotypic analysis of adipose-derived MPC. Representative flow cytometric histograms depicting the co-expression of STRO-1 and CD146 (A) and 3G5 and CD146 in fresh preparations of peripheral adipose-derived single-cell suspensions generated following collagenase/dispase digestion. The STRO-1 and 3G5 antibodies were identified using a goat anti-murine IgM-phycoerythrin while CD146 was identified using a goat anti-murine IgG-fluorescein isothiocyanate.

Figure 12 Developmental potential of purified Adipocyte-derived MPC *in vitro*. Preparations of primary MPC cultures derived from STRO-1⁺/CD146⁺ adipose cells were re-cultured either in standard culture conditions (A), osteogenic inductive medium (B), Adipogenic inductive medium (C) or condrogenic conditions (D) as previously described Gronthos et al. 2003. Following two weeks of multi-differentiation induction, the adipocyte-derived MPC demonstrated the capacity to form bone (B; Alizarin positive mineral deposits), fat (C; Oil Red O positive lipid) and cartilage (D: collagen type II matrix).

Figure 13 Isolation of skin-derived MPC by FACS. Representative flow cytometric histograms depicting the expression of STRO-1, CD146 and 3G5 in fresh preparations of full thickness skin-derived single-cell suspensions generated following collagenase/dispase digestion. The antibodies were identified using either a goat anti-murine IgM or IgG-phycoerythrin. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth medium to assess the incidence of adherent colony-forming cells in each cell fraction.

Figure 14 Clonogenic skin-derived MPC are positive for STRO-1/3G5/CD146. The bar graph depicts the number of adherent colonies recovered from single cell suspensions of enzymatically digested human skin, following fluorescence activated cell sorting, based on their reactivity to antibodies that recognize STRO-1, CD146, and 3G5 (Figure 6), then cultured in standard growth medium as previously described for bone marrow and dental pulp tissue (Shi and Gronthos 2003). The data are expressed as the number of colony-forming units obtained per 10^5 cells plated in the positive and negative cell fractions averaged from two separate experiments.

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Figure 15 Prominent *in vivo* survival of cultured STRO1^{bright} cells adjacent to blood vessels.

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Figure 16 Tumour arteriogenesis induced by cultured STRO1^{bright} cells

15 Figure 17 Tumor arteriogenesis induced by progeny of STRO1^{bright} cells

20 Figure 18 Dose-dependent cardiac arteriogenesis by cultured STRO1^{bright} cells.

Figure 19 Improvement in left ventricular ejection fraction (EF) by myocardial injection of cultured STRO1^{bright} cells.

25 Figure 20 Improvement in left ventricular fractional area shortening (FAS) by myocardial injection of cultured STRO1^{bright} cells.

Figure 21 Improvement in global cardiac function by myocardial injection of cultured STRO1^{bright} cells.

Figure 22. *Ex vivo* expanded STRO-1^{hi} MPC can develop into arterioles *in vitro*. Single cell suspensions of *ex vivo* expanded bone marrow STRO-1^{hi} MPC were prepared by trypsin/EDTA treatment then plated into 48-well plates containing 200 μ l of matrigel. The STRO-1^{hi} MPC were plated at 20,000 cells per well in serum-free medium (Gronthos et al. 2003) supplemented with the growth factors PDGF, EGF, VEGF at 10ng/ml. Following 24 hours of culture at 37°C in 5% CO₂, the wells were washed then fixed with 4% paraformaldehyde. Immunohistochemical studies were subsequently performed demonstrated that the cord-like structures expressed alpha-smooth muscle actin identified with a goat-anti-murine IgG horse radish peroxidase antibody.

Figure 23 A. Immunophenotypic expression pattern of *ex vivo* expanded bone marrow MPC. Single cell suspensions of *ex vivo* expanded bone marrow MPC were prepared by trypsin/EDTA treatment then incubated with antibodies identifying cell lineage-associated markers. For those antibodies identifying intracellular antigens, cell preparations were fixed with cold 70% ethanol to permeabilize the cellular membrane prior to staining for intracellular markers. Isotype matched control antibodies were treated under identical conditions. Flow cytometric analysis was performed using a COULTER EPICS instrument. The dot plots represent 5,000 listmode events indicating the level of fluorescence intensity for each lineage cell marker (bold line) with reference to the isotype matched negative control antibodies (thin line).

Figure 23 B. Gene expression profile of cultured MPC. Single cell suspensions of *ex vivo* expanded bone marrow MPC were prepared by trypsin/EDTA treatment and total cellular RNA was prepared. Using RNazolB extraction method total RNA was isolated and used as a template for cDNA synthesis, prepared using standard procedure. The expression of various transcripts was assessed by PCR amplification, using a standard protocol as described previously (Gronthos et al. 2003). Primers sets used in this study are shown in Table 2. Following amplification, each reaction mixture was analysed by 1.5% agarose gel electrophoresis, and visualised by ethidium bromide staining. Relative gene expression for each cell marker was assessed with reference to the expression of the house-keeping gene, GAPDH, using ImageQuant software.

5 DETAILED DESCRIPTION OF THE ILLUSTRATED AND EXEMPLIFIED
EMBODIMENTS OF THE INVENTION

The invention resides in a method of inducing neovascularisation by use of a composition of precursor cells. This has a range of application.

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The invention thus has application in inducing blood vessel repair or formation, for example in the treatment of cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia, endothelial progenitor cells are administered.

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A wide range of tissues might be treated and such tissues can include, for example, muscle, brain, kidney and lung. Ischemic diseases include, for example, cerebrovascular ischemia, renal ischemia, pulmonary ischemia, limb ischemia, ischemic cardiomyopathy and myocardial ischemia.

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The treatment of these conditions might include the step of isolating mesenchymal progenitor cells from a tissue of the patient, and then readministering them to the patient. The patient may also be treated with compound known to promote formation or repair of blood vessels or to enhance mesenchymal cell growth and/or vascular differentiation. For this purpose the

25 MPCs may be administered to the patient by any suitable means, including, for example, intravensou infusion, bolus injection, and site directed delivery via a catheter.

The invention may also have applicability for the treatment of burns and wounds, including chronic sores such as bed sores, and certain ulcerations. For these conditions the MPCs
30 might be applied topically, perhaps suspended in a cream or togeher with a suitable agent to assist with migration of the cells into the subsurface. Alternatively the MPCs might be held

within a bandage perhaps within a protective matrix that is soluble on prolonged contact with the wound or other surface.

The invention may also have application where a reduced blood supply leads to for example 5 baldness. The composition of MPCs may be injected subcutaneously or dermally in the affected area.

In vitro grown grafts of vascularised tissue are also contemplated by the invention whereby 10 MPCs are grown in media and in the presence of compounds known to promote differentiation into vascular cells, to produce a graft which includes undifferentiation, partially differentiated and some differentiated cells.

In the case of an implant the surgeon may apply a composition containing the MPCs during 15 the implantation of a prosthesis, to promote vascularisation at the interface of the prosthesis and the surrounding tissue. An alternative process may be to have MPCs or a partially or fully differentiated graft developed on the implant. This may or may not be held within a protective matrix. The benefit of a graft is that vascularisation, and thus the healing process may be speeded up.

20 Blood vessels are an ideal position from which to deliver a medicinal product. The discovery of these MPCs and their properties of forming new blood vessels provides an opportunity to delivery over an extended period medicinal products.

The MPCs may be modified to carry various genetic material. The genetic material may be 25 those that encode a variety of proteins including anticancer agents, hormones such as for example insulin, growth factors enzymes cytokines, and the like.

Alternatively the MPCs might be modified to express a blood vessel formation promoter which might assist in the MPCs induction of blood vessel formation, and assisting further with 30 the maintenance of a good vascularisation of the tissue concerned.

For purposes of treating a cardiac vascular disease MPCs may be delivered to the myocardium by direct intracoronary (or graft vessel) injection using standard percutaneous catheter based methods under fluoroscopic guidance, at an amount sufficient for effective therapy. This may be in the range of between 10^4 to 10^7 MPCs. The injection should be made deeply into the 5 lumen (about 1 cm within the arterial lumen) of the coronary arteries (or graft vessel), and preferably be made in both coronary arteries. By injecting the material directly into the lumen of the coronary artery by coronary catheters, it is possible to target MPC rather effectively, and to minimize loss during injection. Any variety of coronary catheter perfusion catheter can be used.

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For the treatment of peripheral vascular disease, a disease characterized by insufficient blood supply to the legs an MPC may be delivered by a catheter that will be inserted into the proximal portion of the femoral artery or arteries, thereby effecting migration of the MPCs to the capillaries of the skeletal muscles receiving blood flow from the femoral arteries. This will 15 provide an angiogenic stimulus that will result in neovascularisation and/or repair of blood vessels in skeletal muscle of the legs.

Compositions or products of the invention for use with coronary or peripheral vascular disease may conveniently be provided in the form of formulations suitable for intracoronary 20 administration. A suitable administration format may best be determined by a medical practitioner for each patient individually. Suitable pharmaceutically acceptable carriers and their formulation are described in standard formulations treatises, e.g., Remington's Pharmaceuticals Sciences by E. W. Martin. Compositions may be formulated in solution at neutral pH, for example, about pH 6.5 to about pH 8.5, more preferably from about pH 7 to 8, 25 with an excipient to bring the solution to about isotonicity, for example, 4.5% mannitol or 0.9% sodium chloride, pH buffered with art-known buffer solutions, such as sodium phosphate, that are generally regarded as safe. The desired isotonicity may be accomplished using sodium chloride or other pharmaceutically acceptable agents such as dextrose, boric acid, sodium tartrate, propylene glycol, polyols (such as mannitol and sorbitol), or other 30 inorganic or organic solutes. Sodium chloride is preferred particularly for buffers containing sodium ions. If desired, solutions of the above compositions may also be prepared to enhance shelf life and stability. The therapeutically useful compositions of the invention are prepared

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by mixing the ingredients following generally accepted procedures. For example, the selected components may be mixed to produce a concentrated mixture which may then be adjusted to the final concentration and viscosity by the addition of water and/or a buffer to control pH or an additional solute to control tonicity.

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For use by the physician, the compositions will be provided in dosage form containing an amount of MPC composition which will be effective in one or multiple doses to induce angiogenesis at a selected level. As will be recognized by those in the field, an effective amount of therapeutic agent will vary with many factors including the age and weight of the patient, the patient's physical condition, and the level of angiogenesis to be obtained, and other factors.

10 The effective doses of the compounds of this invention will typically be in the range of at least about 10^4 MPCs, preferably about 10^6 MPCs, and more preferably about 10^7 MPCs. As noted, the exact dose to be administered is determined by the attending clinician, but is preferably in 15 1 ml phosphate buffered saline.

The presently preferred mode of administration in the case of heart disease is by intracoronary injection to one or both coronary arteries (or to one or more saphenous vein or internal mammary artery grafts) using an appropriate coronary catheter. The presently preferred mode 20 of administration in the case of peripheral vascular disease is by injection into the proximal portion of the femoral artery or arteries using an appropriate arterial catheter.

25 Preferably the MPCs are coadministered with a blood vessel promoting compound. These might include acidic and basic fibroblast growth factors, vascular endothelial growth factor, epidermal growth factor, transforming growth factor α and β , platelet-derived endothelial growth factor, platelet-derived growth factor, tumor necrosis factor α , hepatocyte growth factor, insulin like growth factor, erythropoietin, colony stimulating factor, macrophage-CSF, GM- CSF and nitric oxidesynthase.

30 To further enhance angiogenesis an endothelial progenitor cell modified to express an endothelial cell mitogen may be used. Additionally, an perivascular cell mitogen or a nucleic acid encoding an perivascular cell mitogen can further be administered.

The MPCs might also be injected intramuscularly adjacent a site of the damaged blood vessel.

Composition might include a topical application for wounds and thus may be incorporated into
5 creams lotions and the like.

The MPCs can be delivered in a composition that takes the form of an injectable preparation containing pharmaceutically acceptable carrier such as saline, for example, as necessary. The preparation may require sterilisation and may include stabiliser to maintain a uniform
10 distribution of cells. The final dose of MPCs is preferably in the range of about 10^4 - 10^7 cells.

The present invention relates to mesenchmal precursor cells, in particular those that may be present in the perivascular compartment of vascularised tissue. Such mesenchymal cells may
15 be identified by the presence of the 3G5 surface marker, and perhaps additionally or separately by other early developmental markers such as CD146 (MUC18), VCAM-1 and STRO-1.

Precursor cells are early cells that are substantially at a pre-expansion stage of development. These are cells that have yet to differentiate to fully committed cells, however they need not
20 be stem cells in a strict sense, in that they are necessarily able to differentiate into all types of cells. Partially differentiated precursor cells have a benefit in that they have a greater proliferative potential than stem cells.

The present precursor cells are somewhat differentiated in that they are committed to
25 mesenchymal tissue, as opposed, for example, to haemopoietic tissues. It is evident from the data produced that the MPCs that have been isolated lack markers associated with haemopoietic cells such as CD34, and additionally their differentiation potential does not extend to haemopoietic lines. Additionally they need not necessarily have the potential to differentiate into all mesenchymal cell type, rather, they may be able to differentiate into one,
30 two three or more cell types.

It is anticipated that these precursor cell harvested from the tissues concerned may be useful for regenerating tissue for cells types from which they have been sourced. Thus precursor cells isolated from heart may be reintroduced to regenerate heart tissue, however their potential need not be so limited, precursor cells isolated from one tissue type might be useful for 5 regenerating tissue in another tissue type. The microenvironment in which an undifferentiated cell finds itself is known to exert an influence on the route of differentiation and therefore the reintroduction need not necessarily be tissue specific.

The data presented show that MPCs have been harvested and then re-introduced to produce 10 bone and bone marrow and dentin and pulp respectively, in addition aterioles, cord like structures, have been produced after ex vivo expansion of isolated MPCs.

It is anticipated that a wide range of cells might be produced based on gene expression of markers characteristic for certain cell types. It is thus anticipated that under appropriate 15 culture conditions the range of cell types that can be generated from the perivascular MPCs of the present invention include but are not limited to the following, osteoblast, odontoblast, dentin-producing, chondrocyte, tendon, ligament, cartilage, adipocyte, fibroblast, marrow stroma, osteoclast- and hematopoietic-supportive stroma, cardiac muscle, smooth muscle, skeletal muscle, pericyte, vascular, epithelial, glial, neuronal, astrocyte or oligodendrocyte 20 cell.

One of the benefits of the finding that MPCs can be isolated from perivascular cells is that this greatly expands the range of source tissues from which MPCs can be isolated or enriched and there is no longer an effective restriction on the source of MPCs to bone marrow. The tissues 25 from which these MPCs have been isolated in the exemplifications of this invention are human bone marrow, dental pulp cells, adipose tissue and skin. In addition in situ staining and histological studies have identified that MPC are present in the perivascular compartment of spleen, pancreas, brain, kidney, liver and heart. Given this wide and diverse range of tissue types where perivascular MPCs are present, it is proposed that MPC will also be present from 30 an even wider range of tissue which may include, adipose tissue, teeth, dental pulp, skin, liver, kidney, heart, retina, brain, hair follicles, intestine, lung, spleen, lymph node, thymus, pancreas, bone, ligament, bone marrow, tendon, and skeletal muscle.

These precursor cells of the present invention are distinguished from other known MPCs in that they are positive for 3G5 or perhaps that they carry another perivascular markers. They can be isolated by enriching for an early developmental surface marker present on perivascular 5 cells, in particular the presence of one or more of CD146(MUC18), VCAM-1 and alternatively or additionally high level expression of the marker recognised by the monoclonal antibody STRO-1. Alternatively or additionally enrichment may be carried out using 3G5.

Markers associated with perivascular cells may also be present on the MPCs, for example 10 alpha smooth muscle actin (α SMA).

Other early developmental markers associated with MPCs may also be present. These may include but are not necessarily limited to the group consisting of THY-1, VCAM-1, ICAM-1, PECAM-1, CD49a/CD49b/CD29, CD49c/CD29, CD49d/CD29, CD29, CD61, integrin beta 5, 15 6-19, thrombomodulin, CD10, CD13, SCF, STRO-1bri, PDGF-R, EGF-R, IGF1-R, NGF-R, FGF-R, Leptin-R (STRO-2). Positive expression of one or more of these markers may be used in methods of enriching for MPCs from source tissue.

The MPCs of the present invention may also be characterised by the absence of markers 20 present in differentiated tissue, and enrichment may be based on the absence of such markers.

Similarly it is preferred that the enriched cell populations are not of haemopoietic origin and thus it is preferred that these cells are not present. Markers characteristically identified as not present include but are not limited to CD34, CD45 and glycophorin A. Additional other 25 markers for this purpose might include CD20 and CD19 (B lymphocyte markers), CD117 (c-kit oncoprotein) present on hemopoietic stem cells and angioblasts, CD14 (macrophage), CD3 and CD4 (T cells).

It may be desirable to use the relatively quiescent, directly enriched or isolated perivascular 30 MPCs. Alternatively it has been discovered that expansion of the enriched population can be carried out and have the beneficial effect of resulting in much greater numbers of cells. The effect of expansion of the directly enriched pool of cells is, however, that some differentiation

of the initial MCPs will occur. Expansion over a 5 week period might result in an increase of 10³ fold. Other periods might be chosen to expand the population to between 10² to 10⁵ fold. This potential might be directed by culturing them in media containing cytokines and other factors directing the differentiation to a particular tissue type for example PDGF and VEGF 5 forming smooth muscle alpha cords. These could then be introduced into a tissue with, for example, an insult to assist with repair. Alternatively it may be desired after expansion to re select cells on the basis of an early developmental marker, that might be STRO-1^{bri} to increase the proportion of MPCs in the population.

10 It is found that an essentially pure population of MCPs is not necessary to provide for formation of differentiated cells to form desired tissue structures. The enriched population may have levels of MCPs of greater than about 0.001, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5 or 1% or higher as a proportion of total cell numbers in the enriched population. This order of enrichment can be achieved by the use of a single marker for selection of the enriched MCP 15 population. This is particularly so where the source tissue has an inherently high level of perivascular MCPs. It is found that considerably more 3G5 pos MCPs are present in certain tissue, for example dental pulp, than in bone marrow. Thus in bone marrow 3G5 positive MPCs constitute about 15% of MPC based on STR1^{bri} colony forming cells, whereas in dental pulp that are found to constitute 65% and greater than 90% in fat and skin tissues. Expansion of 20 the population and then re-enrichment using a single marker could result in higher levels of MPCs, perhaps levels greater than about 0.1, 0.5, 1, 2, 5 or 10%

Whilst it is considered desirable that a substantial proportion and preferably a majority of precursor cells are perivascular MPCs, it is not considered essential for certain forms of the 25 invention for perivascular MPCs to be the sole precursor cell form. Other forms of precursors may also be present without unduly interfering with the capacity of the perivascular MPCs to undergo the desired differentiation. Such other forms may include haemopoietic precursors or non-perivascular MPCs, perhaps being negative for 3G5.

30 Certain forms of the present invention provide perivascular MPCs substantially free of endothelial cells. In that context substantially free might be considered to be less than about 5,

2, 1, or 0.1% endothelial cells. Alternatively the context might be an assessment that the enriched population is von Willebrand Factor negative.

It will be understood that recognition of cells carrying the cell surface markers that form the basis of the separation can be effected by a number of different methods, however, all of these methods rely upon binding a binding agent to the marker concerned followed by a separation of those that exhibit binding, being either high level binding, or low level binding or no binding. The most convenient binding agents are antibodies or antibody based molecules, preferably being monoclonal antibodies or based on monoclonal antibodies because of the specificity of these latter agents. Antibodies can be used for both steps, however other agents might also be used, thus ligands for these markers may also be employed to enrich for cells carrying them, or lacking them.

The antibodies may be attached to a solid support to allow for a crude separation. The separation techniques should maximise the retention of viability of the fraction to be collected. Various techniques of different efficacy may be employed to obtain relatively crude separations. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill. Procedures for separation may include, but are not limited to, magnetic separation, using antibody-coated magnetic beads, affinity chromatography and "panning" with antibody attached to a solid matrix. Techniques providing accurate separation include but are not limited to FACS.

It is in the context of these methods that a cell be either negative or positive. The positive cells may either be low(lo) or a hi (bright) expresser depending on the degree to which the marker is present on the cell surface, the terms relate to intensity of fluorescence or other color used in the color sorting process of the cells. The distinction of lo and bri will be understood in the context of the marker used on a particular cell population being sorted.

30 The method of enriching for perivascular MPCs might include the step of making a first partially enriched pool of cells by enriching for the expression of a first of the markers, and then the step of enriching for expression of the second of the markers from the partially enriched pool of cells.

It is preferred that the method comprises a first step being a solid phase sorting step, based on recognition of one or more of the markers. The solid phase sorting step of the illustrated embodiment utilises MACS recognising high level expression of STRO-1. This then gives an enriched pool with greater numbers of cells than if a high accuracy sort was used as a first 5 step. If for example FACS is used first, many of the precursor cells are rejected because of their association with other cells. A second sorting step can then follow using an accurate separation method. This second sorting step might involve the use of two or more markers. Thus in the illustrated embodiment two colour FACS is used to recognise high level expression of the antigen recognised by STRO-1 as well as the expression of CD146. The 10 windows used for sorting in the second step can be more advantageously adjusted because the starting population is already partially enriched.

The method of enriching for perivascular MPCs might also include the harvesting of a source of the stem cells before the first enrichment step using known techniques. Thus the tissue will 15 be surgically removed. Cells comprising the source tissue will then be separated into a so called single cells suspension. This separation may be achieved by physical and or enzymic means.

The preferred source of such perivascular MPCs is human, however, it is expected that the 20 invention is also applicable to animals, and these might include agricultural animals such as cows, sheep, pigs and the like, domestic animals such as dogs, laboratory animals such as mice, rats, hamsters, and rabbits or animals that might be used for sport such as horses.

In a further form the invention might be said to reside a method of generation tissue in a 25 mammal comprising the step of enriching a population of precursor cells as in the first aspect of the invention, and introducing the enriched population into the mammal, and allowing the enriched population to generate the tissue in the mammal.

Another potential use for enriched cells of the present invention is as a means of gene therapy, 30 by the introduction of exogenous nucleic acids for expression of therapeutic substances in the tissue types concerned.

In the context of the present invention the term isolated cell may mean that perivascular MPCs comprise at least 30, 40, 50, 60, 70, 80, or 95% of total cells of the population in which they 35 are present.

EXAMPLE 1 Isolation and expansion of precursor cells

Stem cell niches identified in a number of different adult tissues including skin, hair follicles, bone marrow, intestine, brain, pancreas and more recently dental pulp, are often highly vascularized sites.⁽¹⁾ The maintenance and regulation of normally quiescent stem cell populations is tightly controlled by the local microenvironment according to the requirements of the host tissue.^(2,3) Both the supportive connective tissues of bone marrow and dental pulp contain stromal stem cell populations with high proliferative potentials capable of regenerating their respective microenvironments with remarkable fidelity, including the surrounding mineralized structures of bone and dentin.^(4,5) In the postnatal organism, bone marrow stroma exists as a loosely woven, highly vascularized tissue that supports and regulates hematopoiesis.⁽⁶⁻⁸⁾ At a time when many tissues have lost or decreased their ability to regenerate, adult bone marrow retains a capacity for continuous renewal of haematopoietic parenchymal tissue and is responsible for remodeling the adjoining bone surfaces.^(9,10) In contrast, the inner pulp chamber of teeth is comprised of a non-hematopoietic, compact fibrous tissue, infiltrated by a microvascular network, that is entombed by mineralized dentin.⁽¹¹⁻¹³⁾ Following tooth maturation, dental pulp becomes relatively static, acting only in a reparative capacity in response to a compromised dentin matrix caused by insults such as caries or mechanical trauma.

20

Precursors of functional osteoblasts (BMSSCs: bone marrow stromal stem cells) and odontoblasts (DPSCs: dental pulp stem cells), both forms of MPCs identified by their source tissue, were initially identified by their capacity to form clonogenic cell clusters *in vitro*, a common feature amongst different stem cell populations.^(4,14-18) The progeny of *ex vivo* expanded BMSSCs and DPSCs share a similar gene expression profile for a variety of transcriptional regulators, extracellular matrix proteins, growth factors/receptors, cell adhesion molecules, and some, but not all lineage markers characteristic of fibroblasts, endothelial cells, smooth muscle cells and osteoblasts.^(4,19) However, previous studies have documented that individual BMSSC colonies demonstrate marked differences in their proliferation rates *in vitro* and developmental potentials *in vivo*.^(5,14,20) Similar to these findings, we have recently observed comparable levels of heterogeneity in the growth and developmental capacity of different DPSC colonies.⁽²¹⁾ Together, these studies infer a hierarchical arrangement of

stromal precursor cells residing in bone marrow and dental pulp, headed by a minor population of highly proliferative pluri-potential stem cells that give rise to committed bi- and uni-potential progenitor cell populations.⁽²²⁾

5 Despite our extensive knowledge about the properties of cultured BMSSCs and DPSCs, we still do not know if their *in vitro* characteristics are an accurate portrait of their true gene expression patterns and developmental potentials *in situ*. In addition, it is not formally known if all of the colony-forming cells within each tissue are derived from one pluri-potent stem cell pool or whether they arise from committed progenitors belonging to distinct lineages. There is
10 10 also a lack of information regarding the precise anatomical location of BMSSCs and DPSCs in their respective tissues. This is mainly attributed to the rarity of stem cells and the absence of specific markers that identify different developmental stages during osteogenesis and odontogenesis, particularly for primitive subpopulations. It has previously been hypothesized that one possible niche for precursors of osteoblasts and odontoblasts may be the
15 15 microvasculature networks of bone marrow and dental pulp, respectively.^(23,24)

MATERIALS AND METHODS

Tissue Samples

Iliac crest-derived bone marrow mononuclear cells (BMMNCs), from normal human adult
20 20 volunteers were obtained under guidelines set by the Royal Adealaide Hospital Human Ethics Committee. Normal human impacted third molars were collected from young adults the University of Adelaide Dental Clinic Research under approved guidelines set by the University of Adelaide Human Ethics Committee, respectively. Discarded full thickness skin and peripheral adipose tissue were obtained from routine plastic surgery procedures from the
25 25 Skin Cell Engineering Laboratory, under the guidelines set by the Royal Adelaide Hospital Human Ethics Committee. The pulp tissue was separated from the crown and root as previously described.⁽⁴⁾ Single cell suspensions of dental pulp, skin and adipose tissue were prepared by enzymatic digestion in a solution of 3 mg/ml collagenase type I (Worthington Biochem, Freehold, NJ) and 4 mg/ml dispase (Boehringer Mannheim, GMBH, Germany) for
30 30 one to three hours at 37°C. Single cell suspensions were obtained by passing the cells through a 70 µm strainer (Falcon, BD Labware, Franklin Lakes, NJ). Cell (0.01 to 1 x 10⁵/well) preparations of bone marrow, dental pulp, skin and adipose were then used for either,

immunoselection, RNA extraction, or direct culture in 6-well plates (Costar, Cambridge, MA) as described below.

Other human tissue specimens (Brain, liver, heart, kidney, lung, spleen, thymus, lymph node, pancreas, skin) were obtained from autopsies carried out at the Royal Adelaide Hospital during routine pathological examinations under approved guidelines set by the Royal Adelaide Hospital Human Tissue Committee. Small specimens approximately 0.5 cm² of each tissue type were placed into Tissue-Tek cryomoulds 25 mm x 20 mm x 5 mm (Miles Laboratories; Naperville, IL) and embedded with O.C. compound medium (Miles Laboratories) by immersion into a 150ml to 200ml pyrex glass beaker of pentane (BDH Chemicals, Poole, England) pre-cooled by suspending a glass beaker into a bath of liquid nitrogen. The isopentane has cooled when the bottom of the glass is white. The frozen sections were immediately stored at -80°C. Frozen sections of nerve and muscle tissue were obtained from the Histopathology Department of the I.M.V.S., South Australia and sections of foreskin were obtained from the Immunology Department of the I.M.V.S., South Australia. Sections of formalin fixed, paraffin embedded human foetal limb (52 days) were kindly provided by Dr. T.J. Khong from the Department of Histopathology, Women's and Children's Hospital, Adelaide, South Australia.

Colony Efficiency Assay and Culture

Single cell suspensions were plated at low plating densities (between 1,000 and 10,000 cells per well in triplicates in six well plates) to assess colony-forming efficiency of different immunoselected cell fractions. The cells were cultured in alpha-Modification of Eagle's Medium supplemented with 20% foetal calf serum, 2mM L-Glutamine, 100 µM L-ascorbate-2-phosphate, 100 U/ml penicillin and 10 µg/ml streptomycin at 37°C in 5% CO₂. Day 14 cultures were fixed with 4% formalin, and then stained with 0.1% toluidine blue. Aggregates of equal to or greater than fifty cells were scored as 25 clonogenic colonies equivalent to colony forming units-fibroblastic (CFU-F).

Magnetic-Activated Cell Sorting (MACS)

This procedure is a modification of that described elsewhere.⁽²⁵⁾ Briefly, approximately 1 x 30 10⁸ BMMNCs were incubated with STRO-1bri supernatant (murine anti-human BMSSCs, IgM)⁽²⁹⁾ (1/2) for 1 hour on ice. The cells were then washed with PBS/5% FBS and

resuspended in a 1/50 dilution of biotinylated goat anti-mouse IgM (μ -chain specific; Caltag Laboratories, Burlingame, CA) for 45 minutes on ice. After washing, the cells were incubated with streptavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, F.R.G.) for 15 minutes on ice, then separated on a Mini MACS magnetic column (Miltenyi Biotec) according to the

5 manufacturers recommendations.

Fluorescence activated Cell Sorting (FACS)

STRO-1^{bri} MACS isolated cells were incubated with a streptavidin-FITC conjugate (1/50; CALTAG Laboratories) for 20 minutes on ice then washed with PBS/5% FBS. Single-color

10 fluorescence activated cell sorting (FACS) was performed using a FACStar^{PLUS} flow cytometer (Becton Dickinson, Sunnyvale, CA). Dual color-FACS analysis was achieved by incubating MACS-isolated STRO-1^{bri} BMMNCs with saturating (1:1) levels of CC9 antibody supernatant (mouse anti-human CD146/MUC-18/Mel-CAM, IgG_{2a}, Dr. Stan Gronthos) for one hour on ice. After washing with PBS/5% FBS, the cells were incubated with a second label goat anti-

15 mouse IgG_{2a} (γ -chain specific) phycoerythrin (PE) conjugate antibody (1/50, CALTAG Laboratories) for 20 minutes on ice. The cells were then sorted using the automated cell deposition unit (ACDU) of a FACStar^{PLUS} flow cytometer. Limiting dilution assay: seeded 1, 2, 3 4, 5, & 10 cells per well, 24 replicates, cultured in serum-deprived medium for 10 days as previously described⁽²⁶⁾. Similarly, freshly prepared unfractionated BMMNCs were incubated

20 with CC9 (IgG_{2a}) and 3G5 (IgM) antibodies or isotype-matched negative control antibodies for one hour on ice. After washing with PBS/5% FBS, the cells were incubated with a second label goat anti-mouse IgG_{2a} (γ -chain specific) phycoerythrin (PE) and IgM (1/50; CALTAG Laboratories) conjugated antibodies for 30 minutes on ice. Cells were washed in PBS/5% FBS prior to being analysed using a FACStar^{PLUS} flow cytometer. Positive reactivity for each

25 antibody was defined as the level of fluorescence greater than 99% of the isotype matched control antibodies.

Flow Cytometric Analysis

Single cell suspensions of ex vivo expanded bone marrow MPC were prepared by trypsin/EDTA

30 treatment then incubated with neat STRO-1 supernatant or antibodies identifying different cell line associated markers (10 μ g/ml) for one hour on ice. The cells were then washed in PBS/5% FBS the incubated either with a goat anti-murine IgM-phycoerythrin (1/50, SouthernBiotechnologies), goat

murine or anti-rabbit IgG- phycoerythrin (Caltag Laboratories). For those antibodies identifying intracellular antigens, cell preparations were permeabilize the cellular membrane prior to staining intracellular markers. Isotype matched control antibodies were treated under identical conditions. Flow cytometric analysis was performed using a COULTER EPICS instrument. The dot plots represent:
5 listmode events indicating the level of fluorescence intensity for each lineage cell marker with reference to the isotype matched negative control antibodies.

Immunhistochemistry

Human tissue sections (μm) were de-waxed in xylene and rehydrated through graded ethanol 10 into PBS. Frozen tissue sections (μm) and cytocentrifuge preparations were fixed with cold acetone at -20°C for 15 minutes then washed in PBS. The samples were subsequently treated with PBS containing 1.5% of hydrogen peroxide for 30 minutes, washed then blocked with 5% non-immune goat serum for 1 hour at room temperature. Samples were incubated with primary antibodies for 1 hour at room temperature. Antibodies used: Mouse (IgG₁ & IgG_{2a}) 15 controls (Caltag, Burlingame, CA); Rabbit (Ig) control, 1A4 (anti- α smooth muscle actin, IgG₁), 2F11 (anti-neurofilament, IgG₁), F8/86 (murine anti-von Willebrand Factor, IgG₁) (Dako, Carpinteria, CA); STRO-1; CC9 (anti-CD146); LF-151 (rabbit anti-human dentin sialoprotein; Dr. L. Fisher, NIDCR/NIH, MD). Working dilutions: rabbit serum (1/500), monoclonal supernatants (1/2) and purified antibodies (10 $\mu\text{g}/\text{ml}$). Single staining 20 was performed by incubating the samples with the appropriate secondary antibody, biotinylated goat anti-mouse IgM, IgG₁, IgG_{2a} or biotinylated goat anti-rabbit for one hour at room temperature (Caltag Laboratories). Avidin-Peroxidase-complex and substrate were then added according to the manufacturer instructions (Vectastain ABC Kit standard, Vector Laboratories). Samples were counterstained with hematoxylin and mounted in aqueous 25 media. Dual-fluorescence labeling was achieved by adding the secondary antibodies, goat anti-mouse IgM-Texas Red and IgG-FITC (CALTAG Laboratories), for 45 minutes at room temperature. After washing the samples were mounted in VECTASHIELD fluorescence mountant.

30 Immunomagnetic bead selection

Single cell suspensions of dental pulp tissue were incubated with antibodies reactive to STRO-1 (1/2), CD146 (1/2), or 3G5 (1/2) for 1 hour on ice. The cells were washed twice with

PBS/1%BSA then incubated with either sheep anti-mouse IgG-conjugated or rat anti-mouse IgM-conjugated magnetic Dynabeads (4 beads per cell: Dynal, Oslo, Norway) for 40 minutes on a rotary mixer at 4°C. Cells binding to beads were removed using the MPC-1 magnetic particle concentrator (Dynal) following the manufactures recommended protocol.

5

Matrigel-Arteriole Assay

Single cell suspensions of ex vivo expanded bone marrow STRO-1^{bright} MPC were prepared by trypsin/EDTA treatment then plated into 48-well plates containing 200µl of matrigel. The STRO-1^{bright} MPC were plated at 20,000 cells per well in serum-free medium (Gronthos et al. 2003) supplement

10 with the growth factors PDGF, EGF, VEGF at 10ng/ml. Following 24 hours of culture at 37°C in 5% CO₂, the wells were washed then fixed with 4% paraformaldehyde. Immunohistochemical studies were subsequently performed for alpha-smooth muscle actin identified with a goat-anti-murine IgG hors radish peroxidase antibody/Vectastaining Kit as described above.

15 *Osteogenic, Adipogenic and Chondrogenic Differentiation of MPC in vitro*

Single cell suspensions of ex vivo expanded adipose-derived MPC were cultured in αMEM supplemented with 10% FCS, 100 µM L-ascorbate-2-phosphate, dexamethasone 10⁻⁷ M and 3 mM inorganic phosphate previously shown to induce bone marrow MPC to form a mineralized bone matrix in vitro (Gronthos et al., 2003). Mineral deposits were identified by positive von Kossa staining.

20 Adipogenesis was induced in the presence of 0.5 mM methylisobutylmethylxanthine, 0.5 µM hydrocortisone, and 60 µM indomethacin as previously described (Gronthos et al. 2003). Oil Red O staining was used to identify lipid-laden fat cells. Chondrogenic differentiation was assessed in aggregate cultures treated with 10 ng/ml TGF-β3 as described (Pittenger et al., 1999)

25 *In vivo transplantation studies*

Approximately 5.0x10⁶ of ex vivo expanded cells derived from either STRO-1^{bright}/CD146⁺ BMSCs or CD146⁺ DPSCs were mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer Inc, Warsaw, IN) and then transplanted subcutaneously into the dorsal surface of 10-week-old immunocompromised beige mice (NIH-bg-nu-xid,

30 Harlan Sprague Dawley, Indianapolis, IN) as previously described.⁽⁴⁾ These procedures were performed in accordance to specifications of an approved animal protocol (NIDCR #00-113).

Reverse transcription-polymerase chain reaction.

Total RNA was prepared from STRO-1^{BRT}/CD146⁺ sorted BMMNCs, and control cells (primary BMSSC cultures grown in the presence of 10⁻⁷ M dexamethasone for three weeks) using RNA STAT-60 (TEL-TEST Inc. Friendswood TX). First-strand cDNA synthesis was

5 performed with a first-strand cDNA synthesis kit (GIBCO BRL, Life Technologies) using an oligo-dT primer. First strand cDNA (2 µl) was added to 46 µl of a 1X PCR master reaction mix (Roche Diagnostics, GmbH Mannheim Germany) and 10 pMol of each human specific primer sets: CBFA1 (632bp, and three smaller alternative splice variants)⁽²⁷⁾ sense 5'-
CTATGGAGAGGACGCCACGCCTGG-3' [SEQ ID NO. 1], antisense, 5'-
10 CATA GCCATCGTAGCCTTGCCT-3' [SEQ ID NO. 2]; osteocalcin (310bp)⁽⁴⁾ sense, 5'-
CATGAGAGCCCTCAC-3' [SEQ ID NO. 3], antisense, 5'-AGAGCGACACCCTAGAC-3'
[SEQ ID NO. 4]; GAPDH (800bp)⁽⁴⁾ sense, 5'-AGCCGCATCTCTTTGCGTC-3' [SEQ ID
NO. 5]; antisense 5'-TCATATTGGCAGGTTTCT-3' [SEQ ID NO. 6]. The reactions
were incubated in a PCR Express Hybaid thermal cycler (Hybaid, Franklin, MA) at 95°C for 2
15 minutes for 1 cycle then 94°C/(30 sec), 60°C/(30 sec), 72°C/(45 sec) for 35 cycles, with a final
7 minute extension at 72°C. Following amplification, each reaction was analyzed by 1.5%
agarose gel electrophoresis, and visualized by ethidium bromide staining.

RESULTS

20 *BMSSCs and DPSCs express vascular associated antigens STRO-1 and CD146 in vivo.*

We have previously demonstrated the efficacy of magnetic activated cell sorting (MACS), to isolate and enrich for all detectable clonogenic colonies from aspirates of human marrow, based on their high expression of STRO-1 antigen.^(25,26) To further characterize BMSSCs we incubated the STRO-1^{bright} MACS isolated cells with another monoclonal antibody, CC9,⁽²⁸⁾ that 25 recognizes the cell surface antigen CD146, also known as MUC-18, Mel-CAM and Sendo-1, that is present on endothelial and smooth muscle cells. These studies determined that CC9, selectively bound the STRO-1 bright expressing fraction (STRO-1^{BRT}) from the total STRO-1⁺ population by dual-color FACS analysis (Figure 1A). Cloning efficiency assays using Poisson distribution statistics, yielded a marked increase in the incidence of BMSSCs (1 colony per 5
30 STRO-1^{BRT}/CD146⁺ cells plated), and achieved a 2 x 10³ fold enrichment of the clonogenic colony population when compared to unfractionated marrow (Figure 1B). No colony formation could be detected in STRO-1^{BRT}/CD146⁻ cell fraction (data not shown).

The light scatter properties of STRO-1^{BRT}/CD146⁺ marrow cells were typically larger and more granular than the nucleated erythroid cells and B-lymphocytes comprising the bulk of the STRO-1⁺ population⁽²⁹⁾ (Figure 1C-E). Cytospin preparations of STRO-1^{BRT}/CD146⁺

5 sorted cells were found to be negative for the erythroid (glycophorin-A) and leukocyte (CD45) associated markers (data not shown). Confirmation that BMSSCs represented an early osteogenic precursor population was obtained by RT-PCR analysis of highly purified MACS/FACS-isolated STRO-1^{BRT}/CD146⁺ cells, which failed to detect the early and late 10 osteogenic, markers CBFA1 and osteocalcin, respectively (Figure 1F). However, the progeny of STRO-1^{BRT}/CD146⁺ sorted BMSSCs were found to express both CBFA1 and osteocalcin, following *ex vivo* expansion. Immunolocalization studies demonstrated that the CD146 antigen was predominantly expressed on blood vessel walls in sections of human bone marrow 15 (Figure 1G). Localization of both STRO-1 and CD146 was confined to large blood vessels in frozen sections of human bone marrow trephine (Figure 1H).

15

Immunoselection protocols were subsequently used to determine if human DPSCs also expressed STRO-1 and CD146 *in situ*. The use of either MACS or FACS analysis to isolate DPSCs was restrictive due to the rarity of these cells (1 colony-forming cell per 2×10^3 cells plated) compounded by the limited number of pulp cells (approximately 10^5 cells per pulp 20 sample) obtained following processing. To circumvent this, we pooled several pulp tissues obtained from 3 to 4 different third molars per experiment and employed immunomagnetic bead selection on single cell suspensions of pulp tissue, based on their expression of either the STRO-1 or CD146 antigens. The STRO-1⁺ fraction represented approximately 6% of the total pulp cell population. Comparative studies demonstrated that growth rates of individual 25 colonies were unperturbed in the presence of magnetic beads (data not shown). Colony efficiency assays indicated that the majority of dental pulp derived colony-forming cells (82%) were represented in the minor, STRO-1⁺ cell fraction analogous to BMSSCs (Figure 2). The mean incidence of DPSCs in the STRO-1 positive fraction (329 colony-forming cells per 10^5 cells plated \pm 56 SE, n=3) was six-fold greater than unfractionated pulp cells (55 colony- 30 forming cells per 10^5 cells plated \pm 14 SE, n=3). Using a similar strategy, different fractions of human dental pulp cells were selected based on their reactivity with the antibody, CC9. Colony efficiency assays showed that a high proportion (96%) of dental pulp-derived

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clonogenic colonies were also present in the CD146⁺ population, using immunomagnetic Dynal bead selection (Figure 2). The mean incidence of clonogenic colonies in the CD146⁺ fraction (296 colony-forming cells per 10⁵ cells plated \pm 37 SE, n=3) was seven-fold greater than unfractionated pulp cells (42 colony-forming cells per 10⁵ cells plated \pm 9 SE, n=3).

5

Immunolocalization studies showed that STRO-1 expression was restricted to blood vessel walls and perineurium surrounding the nerve bundles, but was not present in the mature odontoblast layer or fibrous tissue, in frozen sections of human dental pulp tissue (Figure 3A-B). Furthermore, co-localization of CD146 with STRO-1 was detected on the outer blood vessel cell walls, with no reactivity to the surrounding fibrous tissue, odontoblast layer, and the perineurium of the nerve (Figure 3C-D). Importantly, expression of human odontoblast-specific differentiation marker, dentinsialoprotein (DSP), was restricted to the outer pulpal layer containing mature odontoblasts (Figure 3E) and was absent in fibrous tissue, nerve bundles and blood vessels.

10

Differential expression of the perivascular marker 3G5 by BMSSCs and DPSCs.

In the present study, flow cytometric analysis revealed that the cell surface antigen, 3G5, was highly expressed by a large proportion (54%) of hematopoietic marrow cells (Figure 4A).

This observation eliminated 3G5 as a candidate marker for isolating purified populations of

20 BMSSCs directly from aspirates of human marrow. In addition, dual-FACS analysis based on 3G5 and STRO-1 expression was not possible since both antibodies shared the same isotype. Nevertheless, *in vitro* colony efficiency assays for different 3G5/CD146 FACS sorted subfractions demonstrated that only a minor proportion (14%) of bone marrow clonogenic colonies expressed the 3G5 antigen at low levels (Figure 4B). Conversely, a larger proportion 25 (63%) of clonogenic DPSCs (192 colony-forming cells per 10⁵ cells plated \pm 18.4 SE n=3) were present in the 3G5⁺ cell fraction following immunomagnetic bead selection (Figure 2). 3G5 demonstrated specific reactivity to pericytes in frozen sections of human dental pulp tissue (Figure 3F).

30 We next analyzed the expression of more specific markers of endothelial cells (von Willebrand Factor) and smooth muscle cells/pericytes (α -smooth muscle actin) on cytopsin preparations using freshly isolated STRO-1^{BRT}/CD146⁺ BMSSCs and CD146⁺ expressing

DPSCs. A large proportion of purified BMSSCs (67%), were found to be positive for α - smooth muscle actin (Figure 5A), but lacked expression of von Willebrand Factor (Figure 5B). Similarly, the majority of isolated DPSCs (85%) were also found to express α -smooth muscle actin, but not von Willebrand Factor (Figure 5C, 5D). Purified populations of STRO-1^{BRT}/CD146⁺ BMSSCs and CD146⁺ DPSCs were subsequently expanded *in vitro* then transplanted into immunocompromised mice to assess their developmental potentials *in vivo*. The progeny of cultured BMSSCs and DPSCs displayed distinct capacities, capable of regenerating the bone marrow and dental/pulp microenvironments, respectively (Figure 5E, F), and appeared identical to the developmental potential of non-selected multi-colony derived 10 BMSSCs and DPSCs (4).

DISCUSSION

The present study provides direct evidence that two mesenchymal stem cell populations, distinct in their ontogeny and developmental potentials, are both associated with the 15 microvasculature of their respective tissues.

We employed different immunoselection protocols to demonstrate that BMSSCs and DPSCs could be efficiently retrieved from bone marrow aspirates and enzyme digested pulp tissue respectively, based primarily on their high expression of the STRO-1 antigen. This cell surface 20 antigen is present on precursors of various stromal cell types including, marrow fibroblasts, osteoblasts, chondrocytes, adipocytes, and smooth muscle cells isolated from human adult and fetal bone marrow.^(29,32-34) Previous studies have implicated STRO-1 as a marker of pre-osteogenic populations, where its expression is progressively lost following cell proliferation and differentiation into mature osteoblasts *in vitro*.^(27,35,36) The STRO-1 antigen was also found 25 to be present on the outer cell walls of human bone marrow and dental pulp blood vessels, in accord with previous studies that localized STRO-1 on large blood vessels, but not capillaries, in different adult tissues such as brain, gut, heart, kidney, liver, lung, lymphnode, muscle, thymus.⁽⁶⁾ Therefore, STRO-1 appears to be an early marker of different mesenchymal stem cell populations and infers a possible perivascular niche for these stem cell populations *in situ*. 30

To determine if BMSSCs and DPSCs were associated directly with blood vessels we utilized another antibody (CC9),⁽²⁸⁾ which recognizes the immunoglobulin super family member,

CD146 (MUC-18/Mel-CAM), known to be present on smooth muscle, endothelium, myofibroblasts and Schwann cells *in situ*, as well as being a marker for some human neoplasms.⁽³⁷⁾ Notably, CD146 is not expressed by bone marrow hematopoietic stem cells, nor their progenitors. While the precise function of CD146 is not known, it has been linked to 5 various cellular processes including cell adhesion, cytoskeletal reorganization, cell shape, migration and proliferation through transmembrane signaling.

In order to dissect the BMSSC population, STRO-1^{BRT} expressing marrow cells were further distinguished from STRO-1⁺ hematopoietic cells (predominantly glycophorin-A⁺ nucleated 10 erythrocytes) based on their expression of CD146, using dual-FACS analysis. Purified STRO-1^{BRT}/CD146⁺ human BMSSCs displayed light scatter properties characteristic of large granular cells. Our study supports the findings of Van Vlasselaer and colleagues (1994)⁽³⁸⁾ who isolated partially purified BMSSCs from murine bone marrow following 5-fluoracil (5-FU) treatment, and identified this population as having high perpendicular and forward light scatter 15 characteristics. Interestingly, freshly isolated 5-FU resistant murine BMSSCs were also found to be positive for two perivascular markers Sab-1 and Sab-2.⁽³⁸⁾ Conversely, more recent studies have shown that when BMSSCs are cultivated *in vitro*, the most primitive populations display low perpendicular and forward light scatter properties⁽³⁹⁾ and therefore may not reflect the true morphology of BMSSC *in situ*. In the present study, STRO-1^{BRT}/CD146⁺ sorted 20 human BMSSCs lacked the expression of CBFA1 and osteocalcin that identify committed early and late osteogenic populations, respectively,^(40,41) indicating that BMSSCs exhibit a pre-osteogenic phenotype in human bone marrow aspirates. We found that a high proportion of freshly isolated STRO-1^{BRT}/CD146⁺ BMSSCs expressed α -smooth muscle actin, but not the 25 endothelial specific marker von Willebrand Factor, providing direct evidence that this primitive precursor population displays a characteristic perivascular phenotype.

The present study also demonstrated the efficacy of using magnetic bead selection to isolate and enrich for DPSCs directly from human dental pulp tissue based on their expression of either STRO-1 or CD146. Immunolocalization of CD146 appeared to be specific to the 30 microvasculature within dental pulp. Co-localization of both STRO-1 and CD146 on the outer walls of large blood vessel in dental pulp tissue, implied that the majority of DPSCs arise from the microvasculature. However, since the STRO-1 antibody also reacted with the perineurium

in dental pulp and peripheral nerve bundles (unpublished observations), further investigation is required to determine the role of this antigen in neural cell development.

Analogous to BMSSCs, freshly isolated CD146⁺ DPSCs were found to express α -smooth muscle actin but not von Willebrand Factor. DPSCs were also shown to be an immature pre-odontogenic population both by their location distal from the dentin forming surface and by their lack of expression of the human odontoblast-specific dentin sialoprotein (DSP), which is restricted to the outer pulpal layer containing differentiated odontoblasts. We have previously described that *ex vivo* expanded human DPSCs do not express the precursor molecule, dentinsialophosphoprotein (DSPP), *in vitro* when cultured under non-inductive conditions.⁽⁴⁾ Similar studies have shown that DSPP mRNA was highly expressed in freshly isolated odontoblast/pulp tissue, but was not detect in cultured dental papilla cells derived from rat incisors.^(43,44) It is only when DPSCs are induced, either *in vitro*,⁽⁴⁵⁾ or by *in vivo* transplantation to form an ordered dentin matrix that DSPP is expressed.⁽⁴⁾

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In vitro studies of *ex vivo* expanded BMSSCs and DPSCs supported the notion that their progeny were morphologically similar to cultured perivascular cells having a bi-polar fibroblastic, stellar or flat morphology, rather than a polygonal endothelial-like appearance. In addition, we have previously shown that the progeny of BMSSC- and DPSC-derived colonies exhibit heterogeneous staining for both CD146 and α -smooth muscle actin, but lack expression of the endothelial markers, CD34 and von Willebrand Factor, *in vitro*.⁽⁴⁾

The observations that two different mesenchymal stem cell populations such as BMSSCs and DPSCs harbour in perivascular niches may have further implications for identifying stem cell populations in other adult tissues. Recent findings have identified human "reserve" multi-potent mesenchymal stem cells in connective tissues of skeletal muscle, and dermis derived from human fetal and adult samples.⁽⁵⁶⁾ However the exact location, developmental potential and ontogeny of these stem cells is still largely unknown. In the present study, identification of mesenchymal stem cell niches in bone marrow and dentin pulp may help elucidate the fundamental conditions necessary to selectively maintain and expand primitive multi-potential populations *in vitro*, in order to direct their developmental potentials *in vivo*.

EXAMPLE 2

Adult human bone marrow MPC are distinct from stromal precursor cells, haematopoietic stem cells and angioblasts by their high expression of the STRO-1 antigen and lack of CD34 expression

5 Postnatal bone marrow appears to be a hub of residential stem and precursor cell types responsible for blood cell formation (haematopoietic stem cells), endothelial development (angioblast), and connective tissue/stromal differentiation (stromal precursor cells/bone marrow stromal stem cells/mesenchymal stem cells). Recent work by our group (Gronthos et 10 al. 2003; Shi and Gronthos 2003) has, for the first time, purified and characterised human multipotential bone marrow mesenchymal precursor cells (MPC) based on their high expression of the STRO-1 antigen and by their co-expression of the immunoglobulin superfamily members, VCAM-1 (CD106) and MUC-18 (CD146). Early studies by Simmons and Torok-Storb (1991a and b), have shown that bone marrow-derived STRO-1⁺ stromal 15 precursor cells, with the capacity to form adherent colonies in vitro, also expressed the haematopoietic stem cell marker, CD34, albeit at low levels. These studies used CD34 antibody-complement mediated cell lysis to eliminate a high proportion of adherent colony-forming cells in marrow aspirates (Simmons and Torok-Storb 1991b). It is important to note that while the STRO-1 antibody was generated following immunisation of mice with human 20 CD34⁺ bone marrow cells, this may have arisen due to the fact that the STRO-1 antigen is also expressed at moderate to low levels on CD34⁺/Glycophorin-A⁺ nucleated red cells and CD34⁺/CD20⁺ B-lymphocytes. We now offer direct evidence, using sophisticated fluorescence activated cell sorting technology that multipotential adult human bone marrow MPC express high levels of STRO-1, but lack expression to the stromal precursor cell, 25 haematopoietic stem cell and angioblast marker (CD34), the leukocyte antigen (CD45), and the nucleated red cell marker (Glycophorin-A) (Figure 6A-C). These data demonstrate that adult human bone marrow-derived MPC are a novel stem cell population, distinct from more mature stromal precursor cells, haematopoietic stem cells and angioblast (Figure 7).

30 Unless otherwise indicated the materials and methods of this example are the same as those for Example 1.

Figure 6. Expression of CD34, CD45 and Glycophorin-A on STRO-1 positive bone marrow mononuclear cells. Representative histograms depicting typical dual-colour flow cytometric analysis profiles of STRO-1 positive bone marrow mononuclear cells isolated initially by magnetic activated sorting and co-stained with antibodies directed against CD34 (A), CD45 (B) or Glycophorin-A (C). The STRO-1 antibody was identified using a goat anti-murine IgM-fluorescein isothiocyanate while CD34, CD45 and Glycophorin-A were identified using a goat anti-murine IgG- phycoerythrin. The high expressing STRO-1 fraction which contained the clonogenic MPC population was isolated by fluorescence activated cell sorting based on regions R1 and R2.

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Figure 7. Bone marrow MPC are STRO-1 bright, CD34 negative, CD45 negative and Glycophorin-A negative. The graph depicts the results of in vitro adherent colony formation assays performed for each of the different sorted STRO-1 bright populations selected by their co-expression or lack of either the CD34, CD45 or Glycophorin-A antigens, based on regions R1 and R2 as indicated in Figure 6. These data are expressed as the mean incidence of colony-forming units for each cell fraction averaged from two separate experiments.

EXAMPLE 3. *Identification of multipotential MPC in different human tissues*

While the existence and precise location of MPC in different tissues is largely unknown, we

20 have recently demonstrated that MPC appear to reside in a perivascular niche in human bone marrow and dental pulp tissues (Shi and Gronthos 2003). These observations were based on a combination of immunohistochemical and immunoselection methods to identify and isolate different MPC populations based on their expression of the mesenchymal stem cell marker, STRO-1, the smooth muscle and pericyte markers, CD146, alpha-smooth muscle actin and the 25 pericyte specific marker, 3G5. We have now extended these studies demonstrating the co-localization of STRO-1/CD146, STRO-1/alpha-smooth muscle actin, and 3G5/CD146 antigens in a wider variety of tissues including heart, liver, kidney, skin, spleen, pancreas, lymph node (Figure 8).

30 To confirm our earlier findings that MPC can be derived from non-bone marrow tissue such as dental pulp, we used fluorescence activated cell sorting to isolate different MPC populations from adult human peripheral adipose. Single cell suspensions were obtained

following digestion of the adipose tissue with collagenase and dispase as previously described (Shi and Gronthos 2003). The adipose-derived cells were then incubated with antibodies reactive against STRO-1, CD146 and 3G5. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated 5 into regular growth medium (Shi and Gronthos 2003) to assess the incidence of adherent colony-forming cells in each cell fraction (Figure 9). Following 12 days of culture, colonies (aggregates of 50 cells or more) were scored and displayed as the number of colonies per 10^5 cells plated for each cell fraction. Our data demonstrated that MPC can be derived from adipose tissues based on their expression of STRO-1/3G5/CD146 antigens (Figure 10). Dual 10 colour flow cytometric analysis confirmed that only a minor proportion of adipose-derived cells co-expressed STRO-1/CD146 and 3G5/CD146 (Figure 11). These findings are consistent with our previous observations that MPC can be isolated from both bone marrow and dental pulp tissue based on the same set of perivascular markers (Shi and Gronthos 2003). Furthermore, we provide evidence demonstrating that adipose derived MPC isolated by 15 CD146 selection have the capacity to differentiate into different tissues such as bone, fat and cartilage (Figure 12), as previous described (Gronthos et al. 2003).

Recent findings examining the existence of MPC in unrelated tissues such as skin has also been examined to further strengthen our hypothesis. Single cell suspensions were obtained 20 following digestion of full thickness human skin with collagenase and dispase as described above for human adipose tissue. The skin-derived cells were then incubated with antibodies reactive against STRO-1, CD146 and 3G5 identified using either a goat anti-murine IgM or IgG- phycoerythrin. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth 25 medium (Shi and Gronthos 2003) to assess the incidence of adherent colony-forming cells in each cell fraction (Figure 13). Following 12 days of culture, colonies (aggregates of 50 cells or more) were scored and displayed as the number of colonies per 10^5 cells plated for each cell fraction. The data demonstrated that MPC can also be derived from skin based on their expression of STRO-1/3G5/CD146 antigens (Figure 10). Collectively these data suggest that 30 multipotential MPC can be identified and isolated in virtually all vascularised tissues derived from postnatal human tissue based on a common phenotype.

Unless otherwise indicated the materials and methods of this example are the same as those for Example 1.

Figure 8. Reactivity of perivascular makers in different human tissues. Dual-colour immunofluorescence staining demonstrating reactivity of (A) STRO-1 and CD146, (B) STRO-1 and alpha-smooth muscle actin, and (C) 3G5 and CD146, on blood vessels and connective tissue present on spleen, pancreas (Panel I), brain, kidney (Panel II), liver, heart (Panel III) and skin (Panel IV) 20X. The STRO-1 and 3G5 antibodies were identified using a goat anti-murine IgM-Texas Red while CD146 and alpha-smooth muscle actin were identified using a goat anti-murine or IgG-fluorescein isothiocyanate. Co-localization is indicated by overlapping areas of yellow and orange fluorescence (white arrows).

Figure 9. Isolation of adipose-derived MPC by FACS. Representative flow cytometric histograms depicting the expression of STRO-1, CD146 and 3G5 in fresh preparations of peripheral adipose-derived single-cell suspensions generated following collagenase/dispase digestion as previously described (Shi and Gronthos 2003). The antibodies were identified using either a goat anti-murine IgM or IgG-phycoerythrin. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth medium to assess the incidence of adherent colony-forming cells in each cell fraction.

Figure 10. Clonogenic adipose-derived MPC are positive for STRO-1/3G5/CD146. The bar graph depicts the number of clonogenic colonies retrieved from single cell suspensions of enzymatically digested human peripheral adipose tissue, following fluorescence activated cell sorting, based on their reactivity to antibodies that recognize STRO-1, CD146, and 3G5 (Figure 9), then cultured in standard growth medium as previously described for bone marrow and dental pulp tissue (Shi and Gronthos 2003). The data are expressed as the number of colony-forming units obtained per 10^5 cells plated in the positive and negative cell fractions averaged from two separate experiments.

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Figure 11. Immunophenotypic analysis of adipose-derived MPC. Representative flow cytometric histograms depicting the co-expression of STRO-1 and CD146 (A) and 3G5 and

CD146 in fresh preparations of peripheral adipose-derived single-cell suspensions generated following collagenase/dispase digestion. The STRO-1 and 3G5 antibodies were identified using a goat anti-murine IgM-phycoerythrin while CD146 was identified using a goat anti-murine IgG-fluorescein isothiocyanate. Approximately 60% and 50% of the CD146 positive 5 cells co-express STRO-1 and 3G5, respectively. These data suggest that 10% or more of the CD164 positive cells co-express STRO-1 and 3G5.

Figure 12. Developmental potential of purified Adipocyte-derived MPC *in vitro*. Preparations of primary MPC cultures derived from STRO-1⁺/CD146⁺ adipose cells were re-cultured either 10 in standard culture conditions (A), osteogenic inductive medium (B), Adipogenic inductive medium (C) or condrogenic conditions (D) as previously described Gronthos et al. 2003. Following two weeks of multi-differentiation induction, the adipocyte-derived MPC demonstrated the capacity to form bone (B; Alizarin positive mineral deposits), fat (C; Oil Red O positive lipid) and cartilage (D: collagen type II matrix).

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Figure 13. Isolation of skin-derived MPC by FACS. Representative flow cytometric histograms depicting the expression of STRO-1, CD146 and 3G5 in fresh preparations of full thickness skin-derived single-cell suspensions generated following collagenase/dispase digestion. The antibodies were identified using either a goat anti-murine IgM or IgG- 20 phycoerythrin. Cell populations were then selected by FACS, based on their positivity (region R3) or negativity (region R2) to each marker and then plated into regular growth medium to assess the incidence of adherent colony-forming cells in each cell fraction.

Figure 14. Clonogenic skin-derived MPC are positive for STRO-1bri/3G5/CD146. The bar 25 graph depicts the number of adherent colonies recovered from single cell suspensions of enzymatically digested human skin, following fluorescence activated cell sorting, based on their reactivity to antibodies that recognize STRO-1, CD146, and 3G5, then cultured in standard growth medium as previously described for bone marrow and dental pulp tissue (Shi and Gronthos 2003). The data are expressed as the number of colony-forming units obtained 30 per 10⁵ cells plated in the positive and negative cell fractions averaged from two separate experiments.

EXAMPLE 4 *Stro^{bright} Cells Induce Neovascularization (Angiogenesis and Arteriogenesis) And Result In Functional Improvement Of Ischemic Myocardial Tissue*

Figure 1. Engraftment and Survival of Human Stro^{bright} Cells Injected Into Rat Tumors.

5 Athymic nude rats were irradiated with 250 Gy for 5 minutes to remove residual natural killer function, then injected subcutaneously in the flank with 1×10^6 rat glioblastoma cells. Two weeks after implantation, the glioblastoma tumors were directly injected with either 500,000 Stro^{bright} cells, 500,000 Stro^{dim} cells or saline, and animals were sacrificed 7 days later. In 2/3 tumor tissues which received Stro^{bright} cells, staining by immunoperoxidase method using a
10 monoclonal antibody with specific reactivity against human, but not rat, mitochondria, demonstrated numerous human cells around the injection site, indicating medium-term engraftment and survival. Human cells were not detected in any of the three tissues receiving Stro^{dim} cells, suggesting that Stro^{bright} cells might have a survival or replicative advantage in this
15 *in vivo* model system (see panel A). The Stro^{bright} cells were predominantly in clusters nearby small capillaries and arterioles (small arrows) (panel B). In addition, several human cells were seen to incorporate into vascular structures (large arrow) (panel C). These data indicate that human Stro^{bright} cells can both induce neovascularization of endogenous (rat) vessels and can become incorporated into new vessels of human origin.

20 Figure 2. Induction Of Tumor Neovascularization (Angiogenesis And Arteriogenesis) By Human Stro^{bright} Cells. In consecutive sections of the tumor tissue stained by immunoperoxidase method using monoclonal antibodies directed, respectively, against von Willebrand Factor (vWF) and alpha-smooth muscle actin (alpha-SMA), animals injected with Stro^{bright} cells demonstrated significantly greater numbers of capillaries and arterioles (defined,
25 respectively, by vWF staining alone and combined expression of vWF and alpha-SMA) than animals injected with saline.

Figure 3. Stro^{bright} Cells Are More Potent Inducers Of Neovascularization (Angiogenesis And Arteriogenesis) Than Stro^{dim} Cells. Quantitation of arteriolar numbers (defined as vascular
30 structures with lumen diameter > 50 microns and circumferential expression of alpha-SMA) demonstrated that animals injected with Stro^{bright} cells had almost eight-fold greater number of arterioles than saline-treated controls at the site of injection (40 ± 5 vs 6 ± 2 arterioles/high

power field, $p<0.01$), while no difference could be detected distal to the injection site. Animals injected with the Stro^{dim} progeny demonstrated a modest, two-fold increase in the number of arterioles at the injection site relative to saline-treated controls (13 ± 3 vs 6 ± 2 arterioles/high power field, $p<0.01$), indicating that the Stro^{bright} progeny contained the most 5 potent pro-arteriogenic cells following *in vitro* culture.

Figure 4. Dose-Dependent Effect Of Stro^{bright} Cells On Myocardial Neovascularization. To examine whether induction of angiogenesis and arteriogenesis could be extended to other tissues, and was associated with biological significance, cultured progeny of Stro-selected 10 cells were injected by direct intramyocardial injection into the peri-infarct regions of the ischemic hearts in athymic nude rats who had undergone left anterior descending coronary artery (LAD) ligation two days earlier. Animals injected with 1×10^6 Stro^{bright} cells demonstrated three-fold greater numbers of arterioles at the peri-infarct region than animals injected with saline (12 ± 2 vs 4 ± 1 arterioles/high power field, $p<0.01$). In contrast, animals 15 injected with only 0.2×10^6 Stro^{bright} cells, delivered in a total of 1×10^6 unfractionated cultured progeny of Stro-selected cells, induced only 50% greater numbers of arterioles at the peri-infarct region than saline (6 ± 1 vs 4 ± 1 arterioles/high power field, $p<0.05$), indicating that Stro^{bright} cells have a dose-dependent effect on arteriolar induction in the ischemic heart.

20 Figures 5, 6 and 7. Stro^{bright}-Dependent Myocardial Neovascularization Results In Global Improvement of Parameters Of Myocardial Function. We next examined the effects of Stro^{bright}-dependent myocardial neovascularization on global parameters of cardiac function. As shown in figure 5, injection of about $0.1 - 0.2 \times 10^6$ and 1×10^6 Stro^{bright} cells resulted in dose-dependent improvement in ejection fraction (EF) at 2 and 6 weeks, as measured by 25 echocardiography performed and analyzed by a blinded technician. Animals receiving 1×10^6 Stro^{bright} cells demonstrated mean improvement in EF at 2 and 6 weeks of 50% and 75%, respectively, relative to baseline values two days post-LAD ligation. In stark contrast, saline-treated animals showed only 5% mean improvement in EF by 6 weeks ($p<0.01$), and animals treated with Stro-depleted fresh bone marrow mononuclear cells demonstrated no difference 30 compared with those receiving saline. Injection of 1×10^6 Stro^{bright} cells resulted in similar dramatic improvement in fractional area shortening (FAS) (mean improvement of 70% and 90% at 2 and 6 weeks, respectively, figure 6). Stro-depleted bone marrow mononuclear cells

again had no effect, while modest improvement was seen after injection of about 01. - 0.2 x 10⁶ Stro^{bright} cells. Finally, as shown in figure 7, injection of 1 x 10⁶ Stro^{bright} cells resulted in significant improvement in left ventricular compliance compared with saline-treated controls. Animals receiving Stro^{bright} cells demonstrated over 50% reduction in both left ventricular 5 mean end-diastolic pressure and diastolic pressure (each p<0.01), and over two-fold improvement in dp/dt (p<0.01). Together, these results indicate that the neovascularization (angiogenesis and arteriogenesis) of ischemic rat myocardium induced by injection of 1 x 10⁶ human Stro^{bright} cells resulted in significant improvement in both global systolic and diastolic parameters of cardiac function.

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EXAMPLE 5. *Immunophenotypic analysis of ex vivo expanded human bone marrow mesenchymal precursor cells*

We have previously reported that multipotential mesenchymal precursor cells (MPC) can be purified from adult human bone marrow mononuclear cells based on the phenotype STRO-1^{bright}/VCAM-1 (CD106)⁺ or STRO-1^{bright}/MUC-18 (CD146)⁺ (Gronthos et al. 2003; Shi and Gronthos 2003). The MPC population can be readily propagated in vitro under defined culture 15 conditions (Gronthos et al. 2003). We now present data characterising the ex vivo expanded MPC progeny based on markers associated with different cell lineages, at both the mRNA and protein level, using reverse transcriptase-polymerase chain reaction (RT-PCR) and flow 20 cytometric analysis, respectively.

In the first series of experiments, semi-quantitative RT-PCR analysis was employed to examine the gene expression profile of various lineage-associated genes present in the cultured MPC populations (Figure 15). Relative gene expression for each cell marker was assessed 25 with reference to the expression of the house-keeping gene, GAPDH, using ImageQuant software (Figure 15 B). In addition, single-colour flow cytometric analysis was used to examine the protein expression profile of ex vivo expanded MPC based on their expression of cell lineage-associated markers (Figure 15 A). A summary of the general phenotype based on the gene and protein expression of the cultured MPC is presented in Table 1. Direct 30 comparison of the gene expression profile of MPC described in the present patent demonstrated clear differences between this cell population and mesenchymal stem cells (MSC) previously described by Pittenger et al. 1999, (Table 1).

Unless otherwise indicated the materials and methods of this example are the same as those for Example 1.

5 Figure 23 A. Immunophenotypic expression pattern of ex vivo expanded bone marrow MPC. Single cell suspensions of ex vivo expanded bone marrow MPC were prepared by trypsin/EDTA treatment then incubated with antibodies identifying cell lineage-associated markers. For those antibodies identifying intracellular antigens, cell preparations were fixed with cold 70% ethanol to permeabilize the cellular membrane prior to staining for
10 intracellular markers. Isotype matched control antibodies were treated under identical conditions. Flow cytometric analysis was performed using a COULTER EPICS instrument. The dot plots represent 5,000 listmode events indicating the level of fluorescence intensity for each lineage cell marker (bold line) with reference to the isotype matched negative control antibodies (thin line).

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Figure 23 B. Gene expression profile of cultured MPC. Single cell suspensions of ex vivo expanded bone marrow MPC were prepared by trypsin/EDTA treatment and total cellular RNA was prepared. Using RNAzolB extraction method total RNA was isolated and used as a template for cDNA synthesis, prepared using standard procedure. The expression of various
20 transcripts was assessed by PCR amplification, using a standard protocol as described previously (Gronthos et al. 2003). Primers sets used in this study are shown in Table 2. Following amplification, each reaction mixture was analysed by 1.5% agarose gel electrophoresis, and visualised by ethidium bromide staining. Relative gene expression for each cell marker was assessed with reference to the expression of the house-keeping gene,
25 GAPDH, using ImageQuant software.

Figure 23. *Ex vivo* expanded STRO-1^{bri} MPC can develop into arterioles *in vitro*. Single cell suspensions of *ex vivo* expanded bone marrow STRO-1^{bri} MPC were prepared by trypsin/EDTA treatment then plated into 48-well plates containing 200 μ l of matrigel. The
30 STRO-1^{bri} MPC were plated at 20,000 cells per well in serum-free medium (Gronthos et al. 2003) supplemented with the growth factors PDGF, EGF, VEGF at 10ng/ml. Following 24 hours of culture at 37°C in 5% CO₂, the wells were washed then fixed with 4%

paraformaldehyde. Immunohistochemical studies were subsequently performed demonstrated that the cord-like structures expressed alpha-smooth muscle actin identified with a goat-anti-murine IgG horse radish peroxidase antibody.

Table 1. Comparison between cultured human Mesenchymal Precursor Cells (MCP's) and cultured human Mesenchymal Stem Cells (MSC's) following ex vivo expansion. Antigens found to be present on cell surface, intracellular or in the extra cellular matrix. MPCs express 5 markers of tissues with different developmental origin, ie. ECT-ectoderm, MES- mesoderm and END – endoderm.

ANTIGEN	MSC	MPC	Differentiated Cell Type.
STRO-1	-ve	+ve	
Collagen II	-ve	+ve	Chondrocyte (MES)
Collagen IV	-ve	+ve	Fibroblast (MES)
Laminin	-ve	+ve	Fibroblast (MES)
Bone Sialoprotein (BSP)	-ve	+ve	Osteoblast (MES)
Osteocalcin (OCN)	-ve	+ve	Osteoblast (MES)
Nestin	ND	+ve	Neural (ECT)
Glial Fibrillary Acidic Protein (GFAP)	ND	+ve	Neural (ECT)
CBFA1	-ve	+ve	Osteoblast (MES)
Osterix (OSX)	ND	+ve	Osteoblast (MES)
Osteocalcin (OCN)	-ve	+ve	Osteoblast (MES)
Sox9	ND	+ve	Chondrocyte (MES)
Collagen X (COL X)	+ve	+ve	Chondrocyte (MES)
Leptin	ND	+ve	Adipose (MES)
GATA-4	ND	+ve	Cardiomyocyte (MES)
Transferrin (TFN)	ND	+ve	Hepatocyte (END)
Flavin Containing Monooxygenase (FCM)	ND	+ve	Hepatocyte (END)

Table 2. RT-PCR primers and conditions for the specific amplification of human mRNA

Target Gene	Sense/ Antisense (5'-3') Primer Sequences	Product Size
GAPDH	CACTGACACGTTGGCAGTGG/ [SEQ ID NO. 7] CATGGAGAAGGCTGGGGCTC [SEQ ID NO. 8]	417
Leptin	ATGCATTGGGAACCCTGTGC/ [SEQ ID NO. 9] GCACCCAGGGCTGAGGTCCA [SEQ ID NO. 10]	492
CBFA-1	GTGGACGAGGCAAGAGTTCA/ [SEQ ID NO. 11] TGGCAGGTAGGTGTGGTAGTG [SEQ ID NO. 12]	632
OCN	ATGAGAGCCCTCACACTCCTC/ [SEQ ID NO. 13] CGTAGAACGCGCGATAGGC [SEQ ID NO. 14]	289
GFAP	CTGTTGCCAGAGATGGAGGTT/ [SEQ ID NO. 15] TCATCGCTCAGGAGGTCCCT [SEQ ID NO. 16]	370
Nestin	GGCAGCGTTGGAACAGAGGTTGGA/ [SEQ ID NO. 17] CTCTAAACTGGAGTGGTCAGGGCT [SEQ ID NO. 18]	460
GATA-4	GACTTCTCAGAAGGCAGAG/ [SEQ ID NO. 19] CTATCCTCCAAGTCCCAGAG [SEQ ID NO. 20]	800
PDGFβ-R	AATGTCTCCAGCACCTTCGT/ [SEQ ID NO. 21] AGCGGATGTGGTAAGGCATA [SEQ ID NO. 22]	650
Osterix	GGCACAAAGAACCGTACTC/ [SEQ ID NO. 23] CACTGGGCAGACAGTCAGAA [SEQ ID NO. 24]	247
COL X	AGCCAGGGTTGCCAGGACCA/ [SEQ ID NO. 25] TTTCCCACCTCCAGGAGGGC [SEQ ID NO. 26]	387
SOX9	CTC TGC CTG TTT GGA CTT TGT/ [SEQ ID NO. 27] CCT TTG CTT GCC TTT TAC CTC [SEQ ID NO. 28]	598
Ang-1	CCAGTCAGAGGCAGTACATGCTA AGAATTGAGTTA/ [SEQ ID NO. 29] GTTTCCATGGTTTGTCGGCAGTA [SEQ ID NO. 30]	300

References

1. Spradling *et al.*, (2001). *Nature* **414**(6859):98-104.
2. Bianco and Robey (2001) *Nature* **414**(6859):118-121.
- 10 3. Fuchs and Segre (2000) *Cell* **100**(1):143-55.
4. Gronthos *et al.*, (2000) *Proc Natl Acad Sci U S A* **97**(25):13625-30.
5. Kuznetsov *et al.*, (1997). *J Bone Miner Res* **12**(9):1335-47.
6. Bianco *et al.*, (2001) *Stem Cells* **19**(3):180-92.

7. Lichtman (1981) *Exp Hematol* 9(4):391-410.
8. Weiss (1976) *Anatomical Record* 186:161-84.
9. Weiss and Sakai H (1984) *Am J Anat* 170(3):447-63.
10. Dexter and Shadduck (1980) *J Cell Physiol* 102(3):279-86.
- 5 11. Orchardson and Cadden (2001) *Dent Update* 28(4):200-6, 208-9.
12. Peters and Balling (1999) *Trends Genet* 15(2):59-65.
13. Thesleff and Aberg (1999) *Bone* 25(1):123-5.
14. Friedenstein *et al.*, (1974) *Transplantation* 17(4):331-40.
15. Castro-Malaspina *et al.*, (1980) *Blood* 56(2):289-301.
- 10 16. Weissman (2000) *Cell* 100(1):157-68.
17. Uchida *et al.*, (2000) *Proc Natl Acad Sci U S A* 97(26):14720-5.
18. Kuznetsov *et al.*, (2001) *J Cell Biol* 153(5):1133-40.
19. Shi *et al.* (2001) *Bone* 29(6):532-39.
20. Pittenger *et al.*, (1999) *Science* 284(5411):143-7.
- 15 21. Gronthos *et al.*, (2002) *J Dent Res* 81(8):531-5.
22. Owen and Friedenstein (1988) *Ciba Found Symp* 136(29):42-60.
23. Doherty *et al.*, (1998) *J Bone Miner Res* 13(5):828-38.
24. Bianco and Cossu (1999). *Exp Cell Res* 251(2):257-63.
25. Gronthos *et al.*, (1998) Isolation, purification and in vitro manipulation of human
20 bone marrow stromal precursor cells. In: Beresford JN and Owen ME (ed) Marrow
stromal cell culture. Cambridge University Press, Cambridge, UK, pp 26-42.
26. Gronthos and Simmons (1995) *Blood* 85(4):929-40.
27. Gronthos *et al.*, (1999) *J Bone Miner Res* 14(1):47-56.
28. Filshie *et al.*, (1998) *Leukemia* 12(3):414-21.
- 25 29. Simmons and Torok-Storb (1991). *Blood* 78(1):55-62.
30. Canfield and Schor (1998) Osteogenic potential of vascular pericytes. In: Beresford
JN and Owen ME (ed) Marrow stromal cell culture. Cambridge University Press,
Cambridge, UK, pp 128-148.
31. Riminucci and Bianco (1998) The bone marrow stroma in vivo: ontogeny, structure,
30 cellular composition and changes in disease. In: Beresford JN and Owen ME (ed)
Marrow stromal cell culture. Cambridge University Press, UK, Cambridge, UK, pp
10-25.

32. Gronthos *et al.*, (1994) *Blood* **84**(12):4164-73.

33. Oyajobi *et al.*, (1999) *J Bone Miner Res* **14**(3):351-61.

34. Dennis *et al.*, (2002). *Cells Tissues Organs* **170**(2-3):73-82.

35. Stewart *et al.*, (1999) *J Bone Miner Res* **14**(8):1345-56.

5 36. Ahdjoudj *et al.*, (2001) *J Cell Biochem* **81**(1):23-38.

37. Shih (1999) *J Pathol* **189**(1):4-11.

38. Van Vlasselaer *et al.*, (1994) *Blood* **84**(3):753-63.

39. Prockop *et al.*, (2001). *Cytotherapy* **3**(5):393-6.

40. Ducy *et al.*, (1997) *Cell* **89**(5):747-54.

10 41. Komori *et al.*, (1997) *Cell* **89**(5):755-64.

42. Woodbury *et al.*, (2000) *J Neurosci Res* **61**(4):364-70.

43. Dey *et al.*, (2001) *Arch Oral Biol* **46**(3):249-60.

44. Ueno *et al.*, (2001) *Matrix Biol* **20**(5-6):347-55.

45. Couble *et al.*, (2000) *Calcif Tissue Int* **66**(2):129-38.

15 46. Nehls and Drenckhahn (1993) *Histochemistry* **99**(1):1-12.

47. Schor *et al.*, (1995) *Clin Orthop* **313**:81-91.

48. Pugach *et al.*, (1999) *Arkh Patol* **61**(4):18-21.

49. Nehls *et al.*, (1992) *Cell Tissue Res* **270**(3):469-74.

50. Brighton *et al.*, (1992) *Clin Orthop* **275**:287-99.

20 51. Nayak *et al.*, (1988) *J Exp Med* **167**(3):1003-15.

52. Andreeva *et al.*, (1998) *Tissue Cell* **30**(1):127-35.

53. Cattoretti *et al.*, (1993) *Blood* **81**(7):1726-38.

54. Charbord *et al.*, (2000) *J Hematother Stem Cell Res* **9**(6):935-43.

55. Dennis and Charbord (2002) *Stem Cells* **20**(3):205-14.

25 56. Young *et al.*, (2001) *Anat Rec* **263**(4):350-60.

Gronthos *et al.*, (2003). *Journal of Cell Science* **116**: 1827-1835.

Pittenger *et al.*, (1999). *Science* **284**, 143-7.

Simmons and Torok-Storb (1991a). *Blood* **78**(1):55-62.

Simmons and Torok-Storb (1991b). *Blood* **78**:2848.

30 Shi and Gronthos. (2003). *Journal of Bone and Mineral Research*, **18**(4): 696-704.